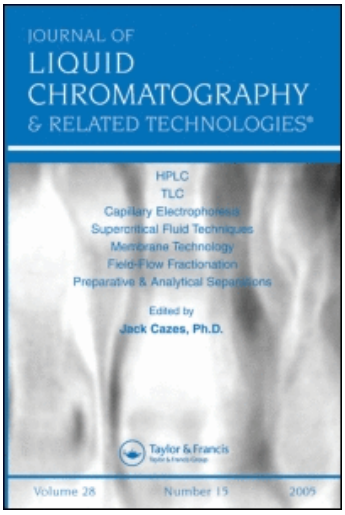

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHY DETERMINATION OF ROGLETIMIDE ENANTIOMERS IN SERUM USING A REVERSED PHASE CELLULOSE-BASED CHIRAL STATIONARY PHASE AND SOLID-PHASE EXTRACTION

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ABSTRACT

A sensitive HPLC method for the quantification of rogletimide enantiomers in serum with UV detection was described. The method involves the use of a solid phase extraction of the R(+) and S (-) enantiomers of rogletimide and the internal standard S(-) amino glutethimide from serum using a C18 Bond-Elute column. Chromatographic resolution of the enantiomers was performed on a reversed phase cellulose-based chiral column (Chiralcel OJ-R) under isocratic conditions using a mobile phase consisting of 80:20 v/v 0.25 M aqueous sodium perchlorate-acetonitrile (pH 5.6 adjusted with perchloric acid) at a flow rate of 0.5 mL/min. Recoveries for R (+) and S (-) rogletimide enantiomers were in the ranges of 84-89% at 200 - 1000 ng/mL level. Intra-day and inter-day precision calculated as % RSD were in the ranges of 3-4% and 1-5% for both Intra-day and inter-day accuracies calculated as % error were in the ranges of 2-4% and 1.5-4% for both enantiomers, respectively. Linear calibration curves were in the concentration ranges of 100-1500 ng/mL for each enantiomer in serum. The limit of quantification of each enantiomer was 100 ng/mL. The detection limit

for each enantiomer in serum using a UV detection set at 257 nm was 50 ng/mL (S/N=2).

INTRODUCTION

The antitumor effects of rogletimide, 3-ethyl-3(4-pyridyl) piperidine-2,6-dione, a new analogue of aminoglutethimide, were examined by Perez et al[1] and Yamamoto et al.,[2] first synthesized by Foster et al[3]. Aminoglutethimide is an agent which is clinically used for the treatment of hormone-dependent tumors acting through inhibition of the aromatase enzyme.[4,5] However, aminoglutethimide produces several neurological side-effects e.g. sedation, ataxia among others, and also interferes with general steroid biosynthesis through inhibition of the desmolase enzyme system (inhibition of side-chain cleavage in cholesterol). Hence, hydrocortisone must be administered as a replacement therapy.[6]

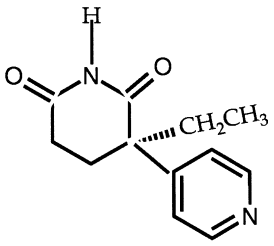
Rogletimide exhibits a strong competitive inhibitory activity which is selective against the aromatase enzyme system while it does not inhibit the desmolase enzyme system, therefore, there is no need for hydrocortisone administration. Furthermore, rogletimide produces little or no neurotoxic side effects.[7]

Rogletimide is currently in clinical trials in postmenopausal women with hormone dependent metastatic breast carcinoma and is considered a more effective and selective chemotherapeutic agent in this respect. Rogletimide is a chiral compound with a asymmetric center at the 3 position (Figure 1) and is clinically administered as a racermic mixture, although it is reported that R(+) enantiomer is more active than S(-) enantiomer.[8]

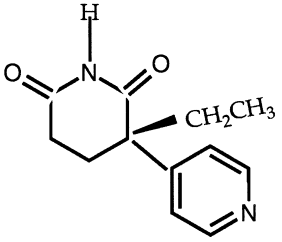
Chemical resolution of racemic rogletimide was previously reported using a chiral stationary phase based on the (R,R) tartaramide.[9] Recently, Aboul-Enein et al.,[10] described a method for the direct resolution of racemic rogletimide using cellulose-based chiral stationary phases (Chiralcel OD and Chirelcel OJ) and operated in a normal phase mode.

Cellulose tris(4-methyl benzoate), known as Chiralcel OJ-R has proven efficient in the direct resolution of many non-steroidal anti-inflammatory drugs of the profen type.[11-14] Many promising results from the Chiralcel OJ-R phase presented by Tanaka et al., included separation and determination of enantiomers of Pantoprazol and other benzimidazole sulfoxides.[15, 16]

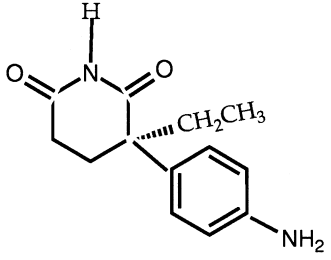
In this paper, we describe a sensitive and stereospecifice assay for the quantification of rogletimide enantiomers in serum using a reversed phase cellulose-based chiral stationary phase (Chiralcel OJ-R) and solid phase extraction. The method is lineal over the range 100-1500 ng/mL using a UV detector set at 257 nm. The detection limit of the method for each enantiomers was 50 ng/mL (S/N = 2).



S (-) - Rogletimide



R (+) - Rogletimide



S(-) - Aminoglutethimide

Figure 1. Chemical structure of rogletimide enantiomers and S(-)-aminoglutethimide (I.S.).

EXPERIMENTAL

Reagents and Chemicals

Powdered samples of R(+) and S(-) rogletimide and the internal standard R(+) aminoglutethimide were kindly supplied by Dr. M. Stogniew (U.S.Bioscience,Inc., West Conshohocken, PA, USA). Blank bovine serum (Cat # 3160-34) was purchased from Instrumentation Lab (Lexington, MA, USA). HPLC grade acetonitrile and 60-62% perchloric acid were obtained from J. T. Baker (Philipsburg, NJ, USA). HPLC grade sodium perchlorate was obtained from Fisher Scientific (Pittsburgh, PA USA) C18 C8, C2, cyanopropyl

and phenyl solid phase extraction columns (100 mg/1cc size) were obtained from Varian Sample Preparation Products (Harbor City, CA, USA). The Vac-Elut vacuum manifold was obtained from Analytichem International (Sunnyvale, CA, USA). All chromatographic solution were filtered through a 0.45 μm filter (Alltech, Deerfield, IL, USA) and degassed by sonication prior to use.

Chromatographic Conditions

Chromatography was performed on an isocratic HPLC system consisting of a Beckman Model 110A solvent delivery module (Beckman, San Ramon, CA, USA) equipped with a 100 μL loop, and spectroflow 757 absorbance detector (Kratos Analytical Ramesy, NJ, USA) set at 257 nm. Data acquisition was performed on a HP Model 3290 integrator (Hewlett-Packard, Avondale, PA, USA).

Separations were accomplished on a reversed phase cellulose-based chiral column (Chiralcel OJ-R, 5 um, 150 mm X 4.6 mm I.D. Chiral Technologies, Eaton, PA, USA) at ambient temperature. The mobile phase consisted of 80:20 v/v 0.25 M aqueous sodium perchlorate-acetonitrile (pH 5.8 adjusted with 60-62% perchloric acid) and was delivered at a flow rate of 0.5 mL/min.

Preparation of Stock and Spiked Standard Solutions

Individual stock solutions of R(+) and S(-) rogletimide and the internal standard R(+)-aminglutethimide were prepared in deionized water to give concentration of 100 μg/mL and storage protected from light at 4°C. Stock solution of rogletimide enantiomers and internal standard were stable for at least two weeks. Appropriate of the R(+) and S(-) rogletimide and the internal standard were pipetted into 1 mL volumetric tubes and serum added to volume to give final concentration of 100, 300, 600, 900, and 1500 ng/mL of each analyte and 5000 ng/mL of the internal standard.

Assay Method

Bond-Elut C18 solid phase extraction (SPE) cartridges were attached to a vacuum manifold and conditioned with 2 column volumes of absolute methanol followed by 2 column volumes of distilled water (Note:- do not allow sorbent to dry). Into the cartridges were transferred blank and spiked serum samples and the vacuum was applied. After the entire serum sample had been aspirated through the cartridge, the cartridge was washed with 4x500 μL of distilled water and then dried under full vacuum for 5 min. The regletimide enantiomers and internal standard were eluted with 4x250 μL of absolute methanol. The eluent was evaporated to dryness under a nitrogen stream at ambient temperature.

The residue was reconstituted in 1 mL of mobile phase and triplicate 100 μL injections were made into the liquid chromatographic system. Linear regression analysis of enantiomer concentration versus peak-height ratio of each rogletimide enantiomer to internal standard produced slope and intercept data which were used to calculate concentration of R(+)-and S(+)- rogletimide enantiomers in each serum sample.

RESULTS AND DISCUSSION

Reversed phase cellulose-based chiral stationary phases employ the same chiral selectors as their normal phase counterparts (e.g. Chiralcel OJ-R vs Chiralcel OJ), but are designed for analytical applications using reversed phase chromatography. Reversed phase chromatographic techniques are preferable in pharmaceutical analysis to normal phase chromatographic techniques since they involve the use of less organic solvents and do not use environmentally harmful solvents such as hexane, chloroform, and methylene chloride. Besides, many drugs exist as salts which are water soluble and are easier to prepare samples for the reversed phase chromatography.

A successful resolution was achieved in the reversed phase mode on the Chiralcel OJ-R column. Initial separation of the enantiomers (Rs=1.5) with retention times of 16-21 min was obtained using a mobile phase consisting of 0.5 M aqueous sodium perchlorate-acetonitrile (70:30 v/v). The influences of buffer type, pH, concentration, and amounts of acetonitrile in the mobile phase on resolution of the analytes of interest were investigated. The function of buffer in a column of this type is to suppress ionization of acidic and basic analytes since ionization of analytes causes deformation and tailing of the analyte peaks. An increase in the concentration of acetonitrile in the mobile phase reduced retention times with loss of some resolution of the analytes of interest.

In our case, whether a buffer or deionized water was used in the mobile phase, dependended on the internal standard used. If the internal standard was an acidic or basic compound, a mobile phase consisting of a buffer- acetonitrile instead of dionized water- acetonitrile as a mobile phase was necessary to prevent the internal standard peak from being deformed. The final mobile phase consisting of 0.25 M sodium perchlorate-acetonitrile (80:20 v/v) provided good peak shape for the internal standard baseline separation of the two enantiomer peaks (Rs = 2.8), suitable retention times (6-8 min. for regletimide enantiomers, and 16 min for the internal standard), and sensitivity to the desired ng/mL range.

Typical HPLC chromatograms for blank serum and serum spiked with 900 ng/mL of each enantiomer and 5000 ng/mL of internal standard are shown in Figure 2. No interferences were observed in blank serum at the retention times of R(+)- and S(-)-rogletimide peaks.

The selection of an internal standard was difficult; some neutral compounds with an amine group in their structure were tested, but only R(+) aminoglutethimide was selected as internal standard based on good recovery for internal standard from serum (87%), suitable retention time (16 min.) and separation factor (α) of 5.3 between R(+)- aminoglutethimide, and the last eluting S(-)- rogletimide enantiomer using a mobile phase consisting of 0.25 M sodium perchlorate- acetonitrile (80:20 v/v). Quantification was based on the plot of concentration of each enantiomer versus peak-height ratios of each rogletimide enantiomers to the internal standard.

The suitability of the system for the separation of the rogletimide enantiomers is shown in Table 1. The retention times of R(+)- and S(-)- rogletimide and internal standard R(+)-aminoglutethimide were 5.9 ± 0.01 , 7.5 ± 0.02 and 15.9 ± 0.01 min respectively (n=9). Relative retention of the R(+)- and S(-)-regletimide enantiomers as expressed by the separation factor α, was calculated to be 2.04. Resolution (Rs) of the R(-)- and S (-)-regletimide enantiomers peaks and the last eluting S(-) enantiomer and the internal standard peaks were 2.8 and 5.3, respectively.

In the course of developing a solid phase extraction procedure for serum sample clean up, three solid-phase extraction cartridges (C18, C8, and cyanopropyl) were investigated. The cyanopropyl cartridge showed interfering endogenous serum peaks at 6.2 and 7.8 min which co-eluted with R(+)- and S (-) rogletimide peaks. An octyl (C8) SPE column was also found to be unacceptable due to co-elution of endogenous serum components with R(+)-rogletimide enaniomers peak at 6.2 min. An octadecyl (C18) SPE column provided the best results in terms of clean-up and recoveries of R(+)- and S(-)-rogletimide enantiomers. Figure 2 shows the chromatogram of blank serum (A) and R (+)- and S (-)- rogletimide added to serum (B).

Table 1

Chromatographic Parameter Data for Rogletimide Enantiomers and Internal Standard in Spiked Serum

Analyte	Rs	α[a]	K^-	T_R,min (mean ± SD, n =6)	N[b]
R(+)rogletimide	2.84	1.62	0.80 ± 0.02	5.9 ± 0.10	---[c]
S(-)rogletimide	5.33	2.04	1.30 ± 0.05	7.5 ± 0.02	1296 ± 47
R(+)aminoglutethimide			2.66 ± 0.03	15.9 ± 0.01	1112 ± 57

[a] Separation factor, calculated as K_2^-/K_1^-. [b] Theoretical plates, calculated as $N = 16 (T_R/w)^2$. [c] Not calculated.

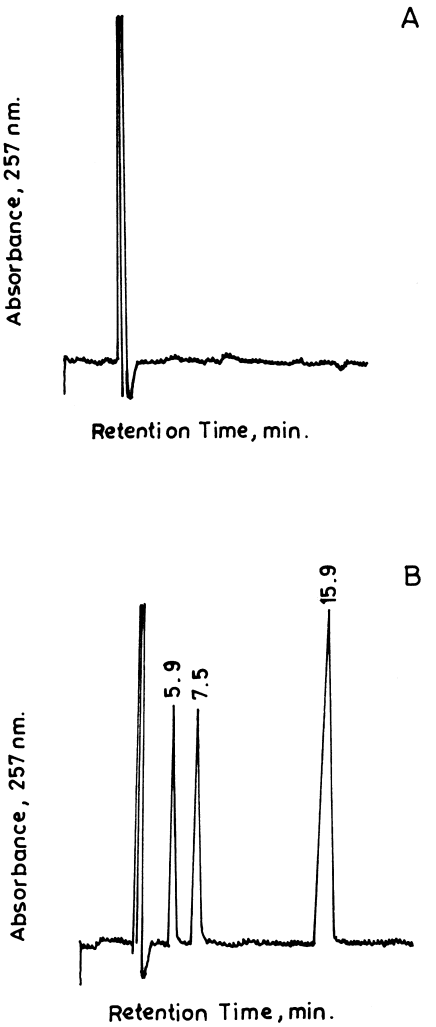


Figure 2. Typical chromatograms of (A) blank serum and (B) serum spiked with R(+) rogletimide (5.9 min), S(-) rogletimide(7.5 min) and internal standard (15.9 min) on a Chiralcel OJ-R column using a a 20:80 v/v acetonitrile-aqueous sodium perchlorate pH 5.8 mobile phase with detection at 257 nm.

Table 2

Absolute Recovery Data for Rogletimide Enantiomers in Serum

Analyte	Conc. Level (ng/mL)	Recovery[a](%) (Mean ± SD n=9)	RSD
R(+)rogletimide	200	88.53 ± 5.93	6.7
	500	84.45 ± 4.21	5.0
	1000	85.96 ± 3.12	3.7
S(-)rogletimide	200	85.03 ± 4.42	5.2
	500	84.69 ± 3.64	4.3
	1000	86.04 ±3.09	3.6

[a] Recoveries were calculated by a comparison of the extracted analyte peak height.

The recoveries of R(+)- and S(-)- rogletimide enantiomers from human serum were assessed by using spiked samples at several concentration levels. The absolute recoveries of R(+)- and S(-)- rogletimide were determined by a comparison of the extracted analyte peak height with the unextracted analyte peak height. The results are shown in Table 2.

Linear calibration curves were obtained in the 100-1500 ng/mL range for each enantiomer of rogletimide. Standard curves were fitted to a first degree polynomial $y = a x + b$, where y is the concentration of rogletimide enantiomer, x is the ratio of drug/internal standard peak heights and a and b are constants. Typical values for the regression parameters a (slope), b (intercept) and correlation coefficient were calculated to be 10.8989, 0.9754, and 0.9993 and 9.0434, 1.0332, and 0.9999 respectively (n = 9). The precision and accuracy (percent error) of the method were determined by using serum samples spiked at 200, 500, 1000 ng/mL levels (Table 3). The data indicate that intra-day precision was in the 3-4% range (n=3) and intra-day accuracy in the 2-4% range (n=3) for both rogletimide enantiomers and that inter-day precision was in the 1-5% range (n=9) and inter-day accuracy in the 1.5-4% range (n=9) or both rogletimide enantiomers.

The minimum detectable concentration of each enantiomer of rogletimide was determined to be 50 ng/mL (S/N=2). The lowest quantifiable level was found to be 100 ng/mL for each enantiomer.

In summary, an HPLC method has been developed and validated for the assay of R(+)- and S(-)- rogletimide enantiomers in serum using a Chiralcel OJ-R column operated in the reversed phase mode. The method utilized a C18 car-

Table 3

Accuracy and Precision Data for Rogletimide Enantiomers in Serum

Analyte	Conc. Added (ng/mL)	Conc Found (ng/mL)	Error (%)	RSD (%)
	200	193.33 ± 5.85[a]	3.3	3.1
Intra-day	500	509.66 ± 15.70	1.9	3.0
R(+)-rogletimide	1000	975.00 ± 32.90	2.5	3.4
	200	195.66 ± 6.65	2.2	3.4
S(-)-rogletimide	500	510.66 ± 19.13	2.1	3.7
	1000	1023.30 ± 36.63	2.3	3.6
Inter-day	200	192.23 ± 3.07[b]	3.9	1.6
R(+)-rogletimide	500	509.16 ± 22.76	1.8	4.5
	1000	986.52 ± 35.16	1.3	3.6
	200	193.16 ± 4.49	3.4	2.3
S(-)-rogletimide	500	507.45 ± 15.92	1.5	3.1
	1000	1017.17 ± 27.23	1.7	2.7

[a] Mean ± SD based on n = 3. [b] Mean ± SD based on n=9.

tridge solid phase extraction for sample clean-up. The procedure is suitable for the separation and quantification of each enantiomer of rogletimide in 100-1500 mg/mL range.

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